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(54) Title: IMMUNOTHERAPY VACCINE FOR MELANOMA TUMORS

(57) Abstract

Immunotherapy vaccines are provided that are composed of disseminated melanoma tumor cell culture antigens and non-toxic, highly effective adjuvants including refined detoxified endotoxin such as monophosphoryl lipid A, and a biological immunostimulant such as mycobacterial cell wall skeleton, trehalose dimycolate, pyridine soluble extract of a microorganism and mixtures thereof, in a pharmaceutically acceptable carrier. By adding tumor antigens to potent but non-toxic immunostimulants, an effective active, specific vaccine for therapeutic and prophylactic purposes is produced. Allogeneic tumor antigens are produced as a cell lysate from a mixture of melanoma cells grown in a tissue culture and thereafter mechanically disrupted. The cell lysate is mixed with an adjuvant to form a vaccine. The vaccine is injected subcutaneously into a host in order to affect a melanoma tumor associated therewith. Cell lysates may be produced from cell lines MSM-M-1, ATCC No. CRL9822, and MSM-M-2, ATCC No. CRL9823.

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IMMUNOTHERAPY VACCINE FOR MELANOMA TUMORS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to tumor vaccines, and more specifically to vaccines comprising a tumor-associated antigen and an adjuvant for treatment of melanoma tumors in a warm-blooded host or patient.

Description of the Prior Art

The incidence of malignant melanoma is increasing steadily, particularly in "sunbelt" states such as those in the American Southwest, and among Caucasians in Australia and Hawaii. This is attributable, at least in part, to an increase in intermittent, intense sun exposure among individuals with fair complexion and those who have predominantly indoor occupations. The current incidence among Caucasians in California and Australia is approximately 15 per 100,000 with double that number in Hawaii. H. Mench and B.E. Henderson, "Cancer Incidence in The Pacific Basin," Nat'l Cancer Inst. Monograph, 1985; 69:105-111.

Surgery has been successful in the early treatment of the primary melanoma tumor, but in its disseminated form melanoma has been resistant to all conventional types of therapy, such as chemotherapy and radiation therapy. Disseminated malignant melanoma tumors are currently treated by methods such as chemotherapy, irradiation, non-specific biological response modifiers, or combinations thereof. Once metastasis has occurred, however, and the cancer had spread or transferred throughout the body, there is little hope for long-term survival of the afflicted host. Chemotherapy is effective in 18 to 20% of patients, with a median duration of response of only about five months. Radiation, except in high dose fractions, is largely ineffective. Chemotherapy, irradiation, or non-specific biological response modifiers are toxic

and have undesirable side-effects that drastically reduce the "quality of life" for the patients. Recent immunological approaches to this disease have included the use of interferon-alpha or interleukin-2, either preceded by cyclophosphamide or together with lymphokine-activated killer cells elicited ex vivo. Deep invasion of melanoma at the time of diagnosis, or involvement of regional lymph nodes, portends a poor prognosis. Adjunctive ("adjuvant") treatment at that time has seemed logical, in an attempt to forestall or perhaps eradicate sites of microscopic metastatic seeding, but none has proved effective thus far.

Active immunotherapy of melanoma with various preparations of whole melanoma cells or lysates has been attempted with some variable degree of success in immunizing the patients, and occasionally in producing desirable clinical remissions. J.C. Bystryn et al., "Preparation and Characterization of a Polyvalent Human Melanoma Antigen Vaccine," J. Biol. Response Mod. 1986;5:211-224; D. Berd et al.,"Potentiation of Human Cell-Mediated and Humoral Immunity by Low-Dose Cyclophosphamide," Cancer Res. 1984; 44:5439-5443; D. Berd et al., "Impairment of Concanavalin A-Inducible Suppressor Activity Following Administration of Cyclophosphamide to Patients with Advanced Cancer," Cancer Res. 1984; 44:1275-1280; P. Livingston et al., "Serological Responses of Melanoma Patients to Vaccine Derived from Allogenic Cultured Melanoma Cells," Int. J. Cancer, 1983; 31:567-575.

In U.S. Application Serial No. 102,909 filed September 30, 1987, by John L. Cantrell for "Vaccine Containing Tumor Antigens and Adjuvants," there is described a vaccine prepared as a mixture of at least one tumor-associated antigen, an adjuvant comprising a refined detoxified endotoxin and other

immunostimulants, in a pharmaceutically acceptable carrier.

The refined detoxified endotoxin utilized by Dr. Cantrell is monophosphoryl lipid A (MPL), disclosed in detail in copending Application Serial No. 732,889, and also in U.S. Pat. No. 4,436,727, issued March 13, 1984, to Edgar E. Ribi for "Refined Detoxified Endotoxin Product." See also E. Ribi et al., "Lipid A and Immunotherapy" Review of Infectious Diseases, Vol. 6, No. 4, July-August 1984, pages 567-572.

The adjuvant also includes an immunostimulant selected from the group consisting of mycobacteri cell wall skeleton, as described in detail in application Serial No. 102,909, and in U.S. Patent No. 4,579,945, issued April 1, 1986 to Steven M. Schwartzman and Edgar E. Ribi for "Purification of Trehalose Dimycolates." See also E. Ribi et al, "Mycobacterial Cell Wall Components in Tumor Suppression and Regression," National Cancer Institute Monograph, No. 39, October 1972, pages 115-120.

The adjuvant further includes trehalose dimycolate, as described in detail in application Serial No. 102,909, and U.S. Patent No. 4,579,945. The adjuvant may also include a purified pyridine soluble extract obtained from a microorganism, as described in detail in U.S. Pat. No. 4,505,903, issued March 19, 1985, to John L. Cantrell, for "Pyridine Soluble Extract of a Microorganism."

The procedure for obtaining tumor-associated antigens is described by J.L. Cantrell in detail in Application Serial No. 102,909, as well as in J.L. Cantrell, et al., "Efficacy of Tumor Cell Extracts in Immunotherapy of Murine EL-4 Leukemia," Cancer Research, 39:1159-1167 April 1979. Various examples of tumor antigens are disclosed in A.E. Reif and

M.S. Mitchell, "Immunity to Cancer," Academic Press Inc., New York 1985, pp. 413-427 and 429-442.

The disclosures of the foregoing patents, publications and references are hereby incorporated herein by reference.

OBJECTS AND SUMMARY OF THE INVENTION

The principal object of the present invention is to provide an improved vaccine for prophylactic and therapeutic treatment of melanoma tumors in a warm blooded mammal host or patient.

A more specific object of the present invention is to provide an effective, nontoxic treatment of melanoma tumors finding principal utility after removal of a primary tumor and at a time when the disease itself is not causing symptoms.

Another object of the present invention is to provide a readily administered melanoma vaccine that has few or no side effects on the host.

Another object of the present invention is to provide a method of inhibiting the growth of melanoma tumors.

Still another object of the present invention is to induce an enhanced immunological response to melanoma tumor associated antigens.

A further object of the present invention is to inhibit the growth of melanoma tumors in a patient or host.

Still a further object of the present invention is to provide an improved adjuvantized vaccine capable of preventing or inhibiting the growth of melanoma tumors.

Still another object of the present invention is to provide a vaccine of the foregoing character that provides some therapeutic benefits even to the extent of retarding growth of tumors in advanced stages of metastasis or growth.

Other objects and advantages of the present invention will become apparent as the following description proceeds.

The present invention is embodied in a vaccine that comprises a mixture of lysates of cultured melanoma cells and an adjuvant. The composition of the melanoma cell mixture is chosen so that it contains a spectrum of tumor antigens, many identifiable by monoclonal antibodies, which could be expected to be present in melanoma cancers from most hosts. The melanoma cells in the form of a lysate (a homogenate of disrupted cells) derived from tissue culture is admixed with an adjuvant and carrier to provide a standardized allogeneic vaccine suitable for immunotherapy of autologous melanomas.

The adjuvants useful in the present invention include refined detoxified endotoxin extract products from selected enterobacteriaceae. The endotoxin extract obtained from these bacteria is hydrolyzed with an acid and lyophilized or freeze-dried to produce a crude detoxified endotoxin component. The crude detoxified endotoxin is then purified and refined to produce the desired refined detoxified endotoxin, a monophosphoryl lipid A (MPL). The endotoxin (MPL) may be combined with cell wall skeleton (CWS) and trehalose dimycolate (TDM) as described in U.S. Patents No. 4,436,727, No. 4,436,728, No. 4,505,900, No. 4,535,386 and No. 4,505,899.

The melanoma cell lysate in admixture with the refined detoxified endotoxin and cell wall skeletonadjuvant provides a vaccine that may be utilized prophylactically or therapeutically to reduce or possibly eradicate melanoma cancers. It is believed that the vaccine works by stimulating the immune system of the host to synthesize specific killer immune cells that are specifically reactive to the tumor antigens.

The vaccine provides external membrane bound, intracellular (inner membrane), and cytoplasmic antigens unaltered by any process. The vaccine can be standardized for its content of certain antigens and therefore is of a reproducible potency.

To illustrate the effectiveness of the vaccine, twenty-two melanoma patients, seventeen with measurable lesions, were treated with the novel vaccine. The vaccine's content of a melanoma-associated antigen was quantitated, its toxicity determined, its effects on antibodies and cell-mediated immunity to melanoma antigens determined, and its influence on the tumors measured. Six or more patients per group were injected subcutaneously with 100, 200 or 400 antigenic units of the vaccine. Five of 17 patients (29.4%) had a remission, two complete and three partial, with three additional minor responses. One complete remission lasted 5-1/2 months, and the patient is alive without disease nearly two years later. Lymph nodes, subcutaneous and pulmonary nodules regressed, whereas liver, adrenal and bone lesions did not. Cytolytic lymphocytes against melanoma cells increased in 12 patients, comprising all eight with a clinical response, while none of seven without an increase had a clinical remission. Antibodies to melanoma antigens increased in 5 patients, all of whom received the same batch of vaccine, and were unrelated to clinical response.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is embodied in an immunotherapeutic vaccine comprising the combination of selected allogeneic melanoma tumor antigens and an effective amount of one or more selected adjuvants. The vaccine is made with a mixture of malignant melanoma cells that have been homogenized to release an immunizing material that boosts cellular immunity by stimulating product of killer thymus derived lymphocytes, or "T-cells." Such cells in turn battle the progression of malignant melanoma tumor cells.

The vaccine is both therapeutic and potentially prophylactic. That is, it can shrink established tumor masses significantly in approximately 20% of patients treated, and may also be effective in preventing the disease from spreading or recurring once it has occurred. It is possible that it may prevent the development of melanoma from precancerous conditions. The purpose of the vaccine is to cause the partial or complete remission of tumor metastases and to prevent the spread of the malignant cells to other parts and sites in the host's body. Several types of T-cells, and probably other immunologically active white blood cells, initiated by the vaccine fight the progression of malignant melanoma cells. The vaccine and adjuvant are effective to stimulate production of such immunologically active blood cells in a warm-blooded animal host or patient.

To produce the vaccine, biopsies tumor fragments of melanoma cells are removed by a biopsy of lesions from several melanoma sources. in vitro cultures of these melanoma cells are started and allowed to grow in an antibiotic-free medium in suitable tissue culture flasks. Fetal bovine serum may be added to concentrations of approximately 10%, together with L-glutamine toconcentrations of about 2 millimolar. The

master seed cultures thus produced are then frozen in liquid nitrogen and stored for future use.

Once removed from the liquid nitrogen storage, the selected amount of seed culture to be used is centrifuged, in a centrifuge tube containing an appropriate amount of growth medium, at 200 x g for five minutes. The supernatant liquid is decanted and the cells are stained to determine viability, counted, and seeded into growth flasks. Aseptic and sterile growth procedures are utilized throughout. Several flasks containing the melanoma cell line are seeded from the initial flasks. After seeking, growth is continued in an incubator at 37°C in 5% CO_2/95% air. The tumor cells are kept in a growth medium lacking serum for 2 to 4 days before harvesting, to remove fetal bovine proteins. The cell monolayers are washed and then harvested. Cell suspensions are frozen in centrifuge tubes. The cells are tested for antigenicity by an appropriate mouse monoclonal antibody specific for the desired antigens. Antigenic units are determined by a binding-inhibition assay based on the enzyme immunoassay.

To produce the vaccine, cells obtained freshly from harvesting or from frozen cell sources stored at 70°C derived from the harvest are mechanically disrupted by a high-speed tissue homogenizer, and the lysates from all cell lines are pooled. Two cycles of freezing and thawing are then performed, to ensure that no active, stable tumor cells remain. The melanoma lysate mixture is tested for sterility, general safety and pyrogenicity, hepatitis contamination, mycoplasma contamination and AIDS virus (HIV) contamination. It is also tested for the presence of viable tumor cells in tissue culture and in nude mice.

The appropriate amount of melanoma lysate and adjuvant, such as the adjuvant product described in

detail in U.S. Pat. No. 4,436,727, and referred to commercially under the tradename "DETOX," are admixed thoroughly. Appropriate pharmaceutically acceptable solutions may be utilized to dilute the vaccine.

The vaccine comprising a mixture of melanoma cell lysate and adjuvant, after thorough mixing, is injected subcutaneously into a human host. Melanoma cell lysates derived from approximately 5 to 40 million cells are used per dose, together with an amount of adjuvant containing about 250 micrograms CWS and about 25 micrograms MPL, emulsified in about 1% to 4% squalene per dose. The ratio of CWS to MPL is generally in the range of about 8 to 12 parts CWS to 1 part MPL. Injections of the vaccine are administered over a period of 6-8 weeks and may be repeated monthly as boosters or periodically as an entire course, if necessary.

The following specific examples will serve to illustrate the present invention.

EXAMPLE I

Preparation of Adjuvant

1. Preparation of Refined Detoxified Endotoxin - Monophosphoryl Lipid A (MPL)

Crude endotoxin is isolated from the polysaccharide-deficient heptoseless Re mutant of Salmonella minnesota (strain R595) by organic solvent extraction. This strain was obtained from the NIH, NIAID, Rocky Mountain Laboratory, Hamilton, Montana. This endotoxin, which consists only of KDO and lipid A, is a glycolipid rather than a typical endotoxic lipopolysaccharide, and is purified by fractional precipitation with organic solvents of appropriate polarities. It is then treated with refluxing 0.1 N hydrochloric acid to yield a complex mixture consisting of free fatty acids and structural homologs of non-toxic monophosphoryl lipid A (MPL). These components

are separated by silica gel column chromatography. Eluted fractions corresponding to structural homologs of MPL, as identified by thin-layer chromatography, are pooled and tested for toxicity. They qualify for experimentation in animals when their 50% lethal dose for intravenously inoculated chicken embryos ($CELD_{50}$) is greater than 10 µg. (The $CELD_{50}$ dose for the parent endotoxin is generally less than 0.01 µg.). The refined detoxified endotoxin has no detectable 2-keto 3-deoxyoctanoate (KDO), between about 500 at about 800 nmoles/mg of phosphorus, and between about 1700 and about 2000 nmoles/mg of fatty acids.

2. Preparation of Mycobacterial Cell Wall Skeleton (CWS)

Cell walls of Mycobacterium bovis, strain BCG obtained from the NIH, NIAID, Rocky Mountain Laboratories, Hamilton, Montana, are prepared with the aid of the Sorvall-Ribi Cell Fractionator (Model RF-1). By using a pressure of 35,000 psi at a temperature of 10-15°C, the mycobacterial cell walls are "cracked," and the protoplasm is exuded in a soluble state. Cell wall envelopes are then harvested by centrifugation and purified by repeated centrifugation and resuspension in water. The cell walls are then treated with RNA-ase and DNA-ase to remove nucleic acids, followed by a series of proteolytic enzymes and a detergent treatment to remove proteins and peptides, respectively. Finally, the preparation is exhaustively extracted with organic solvents to remove "free lipid." The resulting CWS is composed of a polymeric mycolic acid-arabinogalactan-mucopeptide complex.

3. Isolation and Purification of Trehalose-Dimycolate (TDM)

Whole cells of mycobacteria are extracted first with ethanol followed by acetone, and finally with a two-to one mixture of chloroform and methanol (CM 2:1).

The CM extract contains the TDM plus contaminating lipids having lower or higher polarities than TDM. These lipids are selectively separated by precipitating them with compositions of organic solvents in which they are insoluble while retaining the TDM in a soluble phase. The resulting "crude TDM" is purified by silica gel chromatography. Eluted fractions containing pure TDM as determined by TLC are pooled.

4. Pyridine Extraction of Corynebacterium Parvum (PE)

Heat-killed whole cells of C. parvum VPI 0204 obtained from Dr. C. Cummings, Virginia Polytechnic Institute, are extracted twice with pyridine at 37°C and the combined pyridine soluble extracts are concentrated by flash evaporation, dialyzed and lyophilized. The C. parvum 0204 is P. acnes 0204 which is a type II, and results in a PaPE with 37.7% carbohydrate, 4.5% protein and 56.5% fatty acids. The range of same components is broader, and may include about 3% to about 20% by weight protein, about 10% to about 40% by weight carbohydrate, and about 35% to about 70% by weight fatty acids. See Berek, Cantrell, et al., Cancer Research 44:1871-1875. A preferred range is 3-6% PaPE, 15-40% carbohydrate, and 45-70% fatty acids.

5. Mixture of MPL and CWS to form Adjuvant

One illustrative adjuvant is produced by mixing between about 6.25 and about 250 micrograms permilliliter (ug/ml) of MPL and between about 125 and about 750 micrograms per milliliter CWS, preferably in the ration of about 1 to 10 respectively, and specifically in the ration of about 25 µg/ml MPL and about 250 µg/ml CWS.

EXAMPLE II

Production of Melanoma Cell Lysate

In vitro cultures of melanoma cells were initiated from two biopsies of subcutaneous nodules of two different human females. One cell line, designated MSM-M-1 (M-1), was started in 1980 from a biopsy of two lesions on the calf of one human female. This cell line has been deposited with the American Type Culture Collection (ATCC) as designated No. CRL9822. The other, designated MSM-M-2 (M-2), was started in 1981 from a lesion on the back of another human female. This cell line has been deposited with the American Type Culture Collection (ATCC) as designation No. CRL9823. Both of these cell lines are currently available from the laboratory of Dr. Malcolm S. Mitchell at the University of Southern California Cancer Center, 2025 Zonal Avenue, 10-442 GH, Los Angeles, California 90033.

Cell lines M-1 and M-2 were phenotyped with monoclonal antibody W6/32 against HLA-A, B, C antigens and monoclonal antibody Q5/13 against HLA-DR. M-1 was observed to be amelanotic, growing slowly, and expressing HLA-A, B, C (Class 1) antigens and moderately expressing HLA-DR. M-2 was observed to be highly pigmented, growing more rapidly, and strongly expressing HAL-A, B, C, but was devoid of HLA-DR.

Purity of the melanoma cultures was checked regularly for mycoplasma contamination. They were grown in antibiotic-free cultures so that they could be made available to patients without concern about antibiotic sensitivity.

Both M-1 and M-2 were grown to near confluence in RPMI 1640 medium with added fetal bovine serum (10%) and L-glutamine (2 mM), in T-175 and T-75 tissue culture flasks. Approximately 50 and 25 ml,

respectively, of growth medium were used for each flask.

The master seed cultures were frozen in liquid nitrogen in 2 ml freezing vials. The number of cells per vial averaged 5 million. Once removed from liquid nitrogen, the vials were warmed in a 37°C water bath until completely thawed (about 5 min.). The vial contents were transferred to centrifuge tubes containing 30 ml of growth medium, centrifuged at 200 x g for 5 min., and the supernatant fluid decanted. Cells were stained for viability, counted, and seeded into four T-175 flasks containing 50 ml of growth medium at approximately 10^5 cells per ml. Cells were grown to confluence at 37°C in an atmosphere containing 4-7% CO_2. Aseptic technique and sterile, depyrogenated glassware was used for all growth procedures.

Four confluent T-175 flasks were treated with 25 ml of Dulbecco's BS containing 0.02% EDTA and incubated form 10 min. The fluid with cells was poured into four 50 ml Falcon tubes, which were centrifuged at 200 x g for 10 min., and the supernatant liquid decanted. The contents of all four tubes were combined and resuspended in 8 ml RPMI 1640 medium. 500 ml RPMI 1640 medium were added to an aspirator bottle. The 8 ml of RPMI 1640 cell suspension was then added to it with agitation. Eight milliliters of the suspension were then removed and added to 17 ml of RPMI 1640 medium in a T-75 flask, which was used as a control flask to monitor cell growth. An additional 1500 ml RPMI 1640 medium was then added to the 500 ml in the aspirator bottle. The two-liter cell suspension, contained in the aspirator bottle fitted with sterile tubing, was used to seed one ten-tiered flask called a Cell Factory . Each Cell Factory was seeded with approximately 10^8 cells.

The Cell Factories were placed in a CO_2 incubator at 37°C for 5 to 9 days. The medium was decanted, and replaced with 2 liters of serum-free medium per each Cell Factory. Growth continued in the incubator for 2 4 days at 37°C. M-1 and M-2 cell line growth was characterized by opaque monolayers on the lower side of the flasks or Cell Factories.

The medium was decanted from the Cell Factory and the cells were washed with 2 liters of PBS to remove any remaining medium, dead cells, and fetal bovine serum residues. Next, 2 liters of PBS containing at 0.02% EDTA were added and the cells were incubated at 37°C for 45 min. or until cells detached from the vessel surface. Remaining cells were loosened with regular gentle "slaps" of the Cell Factory prior to harvest.

At the end of the incubation period, the contents of the Cell Factory were centrifuged at 250 x g for 10 min. The supernatant fluid was decanted, the cells resuspended in 20 ml PBS, and centrifuged at 250 x g for 10 min. The supernatant fluid was again decanted and the cells were resuspended in a final volume of 20 ml PBS. A cell count was taken. Approximately 10^9 cells were harvested from each Cell Factory.

The cell suspensions were frozen at -70°C in 50 ml centrifuge tubes containing 20 ml PBS (one tube per Cell Factory).

The contents of the M-1 and M-2 Cell Factories were tested for antigenicity by measuring the amount of one melanoma-associated antigen (P250) using mouse monoclonal antibody (Mab) 9.2.27 specific for that antigen. The antibody was supplied by Dr. Ralph Reisfeld of the Scripps Clinic and Research Foundation, LaJolla, California.

A binding-inhibition assay based on the enzyme immunoassay was used to determine antigenic units

(a.u.). One hundred fifty µl of mouse monoclonal antibody Mab 9.2.27, labelled with horseradish peroxidase, were incubated with 30 µl of melanoma lysate in a medium of PGS, 1% BSA (bovine serum albumin) for 1 hour at 37°C. Fifty µl of this mixture were added in triplicate to target melanoma cells fixed on polyvinyl Falcon microtiter wells and incubated for 1 hour at 37°C. After washing, the peroxidase substrate solution containing o-phenylenediamine was added. After 30 minutes the reaction was stopped and the absorbance measured with an EIA plate reader at 490 nm.

A standard binding curve was constructed with a melanoma lysate derived from an abdominal tumor. From this curve, the antigenic content of a test preparation was determined. One antigenic unit (a.u.) is defined as the amount of antigen present in 10 µl of undiluted standard melanoma extract that bound maximally (at least 20% inhibition) to Mab 9.2.27. A computerized program called "Curve-fit" developed by Jeffrey S. Mitchell of Epstein, Mitchell, and Blackford Computer Consultants of New Haven, Connecticut was used to calculate the number of antigenic units in each new lot of melanoma lysate directly from the equation described by the standard curve. In addition, the number of tumor cell equivalents was noted. Approximately 107 cells are equivalent to 100 a.u.

EXAMPLE III

Vaccine Preparation

Cells from Example II, frozen at -70°C, were thawed over ice. The cells were mechanically disrupted with a Polytron stainless steel high-speed tissue homogenizer (Tekmar Co., Cincinnati, Ohio. The cells were pulsed repeatedly until no intact cells could be detected under the microscope. The cells from each Cell Factory were disrupted independently. Then the

lysates from both cell lines and all Cell Factories were pooled. After pooling, lysate concentration was adjusted to 20 x 10^6 T.C.E. (tumor cell equivalents) per ml by adding PBS to the cell lysates.

The lysate was kept in an ethanol/ice bath throughout the dispensing procedure. A 50-ml plastic tube was filled with 25 ml of the cell lysate and 1 ml aliquots were injected into 2 ml sterile, empty pyrogen-free vials with a 1- ml syringe using a 25-gauge needle. The tube was hand-shaken before each filling of the syringe. The top of each vial was wiped with an alcohol spongette. The vials were kept on ice, then frozen at -70°C. Other appropriate fill methods may be utilized.

The melanoma vaccine lysate was tested for sterility under FDA guidelines and for pyrogenicity by American McGaw Biological Test Center, Irvine, California, and found acceptable.

The melanoma vaccine lysate was tested for HIV (AIDS virus) contamination in the laboratory of Dr. Suray Rasheed at the University of Southern California, and found acceptable.

The melanoma vaccine lysate was tested for general safety and pyrogenicity at American McGaw Biological Test Center, Irvine, California and found acceptable.

The melanoma vaccine lysate was tested for hepatitis contamination at the University of Southern California, Serology Laboratory, and found acceptable.

The melanoma vaccine lysate was tested for mycoplasm contamination at Coriell Institute for Medical Research, Camden, New Jersey, and found acceptable.

Immediately prior to injection, the melanoma vaccine lysate was mixed thoroughly with the 10:1 CWS/MPL adjuvant. Each 1 ml vial of lysate at a

concentration of 200 a.u./ml was mixed with 0.25 ml CWS/MPL adjuvant.

EXAMPLE IV

Use of Vaccine and Results

Patients received approximately 10, 20 and 40 million tumor cell equivalents, i.e., 100, 200 or 400 antigenic units, of the vaccine mixed with the adjuvant DETOX comprising 250 µg CWS and 25 µg MPL. One antigenic unit is defined as the amount of antigen present in 10 µl of undiluted standard melanoma extract that bound maximally (80% of greater in inhibition) to mouse monoclonal antibody 9.2.27. The dose was injected subcutaneously into the deltoid region or buttocks of 17 patients with disseminated melanoma and 5 patients with a high risk of recurrence but with no measurable metastasis. At least 6 patients received each dose level of vaccine (100, 200 or 400 antigenic units). One patient with a positive skin test to the vaccine received only 50 antigenic units.

By physical examination, plain x-rays or CT scans, the longest perpendicular diameters of tumor masses that were chosen as sentinel lesions were measured at weekly intervals during and for 2 weeks after treatment. Strict standard definitions involving both degree and duration of response were used to categorize a clinical response as a complete remission, partial remission, minor response or no response. Thus, a "complete remission" was a disappearance of all measured lesions for at least 4 weeks; a "partial remission" was a decrease of 50% in the sum of the products of greatest perpendicular diameters of all measured lesions, lasting at least 4 weeks; a "minor response" was a decrease of 25%-50% in the sum of the products of greatest perpendicular diameters of all measured lesions, or 50% decrease for less than

4 weeks; and "no response" was any response less than a minor response.

There was no toxicity of the treatment, except for slight soreness at the sites of injections one day later. Granulomas formed at several sites of injection in two patients who were given injections that were too superficial. No systemic side effects were observed, and a variety of standard hematological and chemical blood tests that were performed weekly showed no evidence of toxicity to vital organs. Likewise, no adverse immunological affects such as rash or anaphylaxis were seen.

The immunological effectiveness of the vaccine was observed in several ways, the most dramatic of which was the shrinkage of measurable lesions in several patients. As shown Table 1, "Summary of Phase I Trials," the active immunotherapy induced complete or partial remission of melanoma in 5 of the 17 patients (29.3%) who had measurable lesions. Two patients had complete regression of their disease and 3 others, partial remission. Minor responses were also noted in three other patients, including more than 50% regression of large nodules in one patient, but which did not last 4 weeks. Nine patients had no response, i.e., progressive disease despite treatment.

Of most interest were the two complete responses. Patient L.A., a 30-year-old woman, was given 200 a.u. of vaccine weekly, and began to have shrinkage of the numerous subcutaneous nodules on her right thigh and buttock within 1-week after the first dose. This rapid onset of resolution was common to all patients who responded to the 200 a.u. dosage, whereas at the 50 or 100 a.u. level, regression began 3 to 5 weeks after the beginning of treatment. Many of L.A.'s nodules were impalpable within 3 weeks and all were gone within six. The patient had a 4 cm necrotic nodule, which was

TABLE I
Summary of Phase I Trials

Patient	Dosage in a.u.	Cytolytic Lympho-cytes(a)	Skin Test(b)	Ab by EIA	Re-sponse
C.C.	50 (M-1)	+; 22; 29	+--++	+	PR
L.S.	100 (M-1)	0	0--0	+	None
N.C.	100 (M-1)	+; 15; 15	0--+	+	None
S.O.	100+CY (M-1)	+; 15; 22	0--+	+	NE
T.B.	100+Cy (M-1)	+; 7; 7	0--0	+	MR
E.C.	100+Cy	+; 43; 43	0--0	0	NE
T.W.	200	+; 43; 43	0--0	+(c)	MR
E.L.	200	0	0--0	0	None
L.A.	200	+; 7; 92	0--0	+(c)	CR
B.R.	200	0	0--0	0	None
L.L.	200	0	0--0	0	None
M.R.	200+Cy	+; 43; 43	0--0	0	PR
K.D.	200+Cy	+; 43; 43	0--0	0	None
T.S.	200+Cy	0	0--0	0	None
J.B.	200+Cy	+; 22; 22	0--0	+(c)	CR
D.C.	200+Cy	0	0--0	0	None
J.S.	400	0	0--0	0	NE
B.N.	400	0	0--0	0	NE
W.M.	400	0	0--0	0	NE
G.M.	400+Cy	+; 8; 15	0--0	0	PR
G.G.	400+Cy	+; 8; 15	0--0	0	MR
G.F.	400+Cy	0	0--0	0	None

(a): + = Elevation noted: n1 = day rise first noted; n2 = day peak noted.
(b): 0--+, conversion to positive; +--++, increase greater than 25% over original area.
(c): Antibody present before treatment but not significantly increased afterwards.

Abbreviations: a.u., antigenic units; EIA, enzyme immunoassay; CR, complete remission; PR, partial remission; MR, minor response; NE, not evaluable (no measurable disease).

slowly diminishing, excised during the fifth week because it was causing discomfort when she was seated. When the resolution first began, her skin felt indurated, but over several months, it gradually regained its former texture. She had a single new 5 mm lesion in the skin of the right lower quadrant, just above the inguinal ligament, which was immediately resected 5-1/2 months after the remission began, and another of the same size at 10 months. She otherwise remained free of disease for nearly 2 years, as verified by normal CT scans of the lung, liver and brain.

Patient J.B. had had resections of large lymph nodes involved with melanoma from his right axilla for the 3 months prior to his entering the study, having had ostensibly radical resection of axillary disease before that. He had measurable shrinkage of his only lesion, a 2.5 cm diameter lymph node, one week after the first injection, which continued weekly until it disappeared entirely on physical examination and on CT scan by the 6th week. During the 8 weeks of study, and for 5-1/2 months thereafter, no new lymphadenopathy appeared. Retroperitoneal lymphadenopathy was found on a CT scan at that time, and he was considered off-study.

The partial remissions were no less gratifying because the patients in question had bulky or numerous subcutaneous or lymph nodal tumor masses. Patient C.C. had lesions scattered over a region on her right lower extremity calf to groin, measuring from 0.8 to 2.2 cm in diameter. All lesions shrank during the one-week respite after the fourth injection, and most remained small until the middle of a second course of vaccine, 5 or 6 weeks later. Two nodules became impalpable within 3 weeks. Biopsy of a remaining nodule showed considerable necrosis of tumor cells, with some

peripheral lymphocytic infiltration. During both courses, especially the second,t he nodules fluctuated in size, with a peculiar and thus far unexplained 3 or 4 day periodicity. Patient C.C. was given the lowest dose on the study, 50 a.u. weekly, because she was the only one to have a positive melanoma skin test before therapy.

Patient M.R. with 3 large (4-5 cm) subcutaneous nodules, and masses in the left adrenal gland and the lung, received a course of 200 a.u. per week, preceded by low-dose cyclophosphamide. He noted a rapid softening of all skin nodules within a week, and by measurements on day 8 the lesions were indeed smaller and flatter. All reached their nadir within a month, and one became impalpable by the end of therapy. The adrenal and lung nodules were stable during that time.

A third patient, G.G., had a flattening of his many, approximately 1 cm, subcutaneous nodules at the sixth week, on 400 a.u. per week preceded by cyclophosphamide. On the seventh week he had a clear shrinkage of all five sentinel lesions to an aggregate of less than 50% their original size.

Patient T.B. had more than 50% shrinkage of subcutaneous and lymph node masses that began 3 weeks after the onset of therapy with low dose cyclophosphamide and 100 a.u. of vaccine weekly, but which lasted only 3 weeks. However, one of this patient's subcutaneous nodules became and has remained impalpable. He then received cyclophosphamide + IL-2, has had a persistent PR, and is now on maintenance therapy 18 months after beginning immunotherapy. Patient T.W. had 4 large subcutaneous metastases, one of which began to shrink within 1 week, diminishing from 1.8 cm in diameter to 0.75 cm, and remained at 5 mm size thereafter. Since the other 3 subcutaneous masses and lesions in the liver did not shrink, his

aggregate response was less than a PR. Patient G.G. had approximately 30% regression of his subcutaneous nodules lasting only 3 weeks.

The herein described invention is disclosed in further detail in M.S. Mitchell et al. "Active Specific Immunotherapy for Melanoma: Phase I Trial of Allogeneic Lysates and Novel Adjuvant," published Cancer Res. 48, 5883-5893, October 15, 1988, which publication and disclosure therein is hereby incorporated by reference.

While a certain illustrative embodiment of the present invention has been described in detail, it should be understood that there is no intention to limit the invention to the specific form and method disclosed. On the contrary, it is the intention to cover all modifications, equivalents, and uses falling within the spirit and scope of the invention as expressed in the appended claims.

CLAIMS

1. An immunotherapeutic melanoma tumor vaccine comprising:

a melanoma cell lysate produced from allogeneic melanoma tumor cells; and an adjuvant comprising a refined detoxified endotoxin and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose dimycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

2. The vaccine of Claim 1 wherein said refined detoxified endotoxin has no detectable 2-keto-3-deoxyoctanoate, between about 500 and about 800 nmoles/mg of phosphorus, and between about 1700 and about 2000 nmoles/mg of fatty acids.

3. The vaccine of Claim 1 wherein said refined detoxified endotoxin is monophosphoryl lipid A.

4. The vaccine of Claim 1 wherein said immunostimulant is mycobacterial cell wall skeleton.

5. The vaccine of Claim 1 wherein said immunostimulant is trehalose dimycolate.

6. The vaccine of Claim 1 wherein said immunostimulant is a pyridine soluble extract of a microorganism.

7. The vaccine of Claim 1 wherein said immunostimulant is a combination of mycobacterial cell wall skeleton and trehalose dimycolate.

8. The vaccine of Claim 1 wherein said immunostimulant is a combination of mycobacterial cell wall skeleton and a pyridine soluble extract of a microorganism.

9. The vaccine of Claim 1 wherein said immunostimulant is a combination of trehalose dimycolate and a pyridine soluble extract of a microorganism.

10. The vaccine of Claim 1 wherein said immunostimulant is a combination of mycobacterial cell wall skeleton, trehalose dimycolate and a pyridine soluble extract of a microorganism.

11. The vaccine of Claim 1 wherein said pyridine soluble extract of a microorganism contains between about 3% and about 20% by weight of protein, between about 10% and about 40% by weight of carbohydrate, and between about 35% and about 70% by weight of fatty acids.

12. The vaccine of Claim 1 wherein said pyridine soluble extract of a microorganism contains about 4.5% protein, about 37.7% carbohydrate and about 56.5% fatty acids.

13. A process for the treatment of a melanoma tumor on a patient, which comprises administering to said patient an anti-tumor effective amount of a vaccine comprised of a melanoma cell lysate produced from allogeneic melanoma tumor cells, and an adjuvant comprising a refined detoxified endotoxin, and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose dimycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

14. The process of Claim 13 wherein said refined detoxified endotoxin has no detectable 2-keto-3 deoxyoctanoate, between about 500 and about 800 nmoles/mg of phosphorus, and between about 1700 and about 2000 nmoles/mg of fatty acids.

15. The process of Claim 13 wherein said refined detoxified endotoxin is monophosphoryl lipid A.

16. The process of Claim 13 wherein said immunostimulant is trehalose dimycolate.

17. The process of Claim 13 wherein said immunostimulant is trehalose dimycolate.

18. The process of Claim 13 wherein said immunostimulant is pyridine soluble extract of a microorganism.

19. The process of Claim 13 wherein said immunostimulant is a combination of mycobacterial cell wall skeleton and trehalose dimycolate.

20. The process of Claim 13 wherein said immunostimulant is a combination of mycobacterial cell wall skeleton and a pyridine soluble extract of a microorganism.

21. The process of Claim 13 wherein said immunostimulant is a combination of trehalose dimycolate and a pyridine soluble extract of a microorganism.

22. The process of Claim 13 wherein said immunostimulant is a combination of mycobacterial cell wall skeleton, trehalose dimycolate, and a pyridine soluble extract of a microorganism.

23. The process of Claim 13 wherein said pyridine soluble extract from said microorganism contains between about 3% and about 20% by weight of protein, between about 10% and about 40% by weight of carbohydrate, and between about 35% and about 70% by weight of fatty acids.

24. The process of Claim 13 wherein said pyridine soluble extract of a microorganism contains about 4.5% protein, about 37.7% carbohydrate and about 56.5% fatty acids.

25. A process for the treatment of a melanoma tumor in a host, which comprises administering to said host an anti-tumor effective amount of a vaccine comprised of a melanoma cell lysate produced from allogeneic melanoma tumor cells and an adjuvant comprising a refined detoxified endotoxin, and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton,

trehalose dimycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

26. A vaccine useful for the treatment and prevention of melanoma tumors in a host, said vaccine comprising a melanoma cell lysate produced from an in vitro culture of allogeneic melanoma tumor cells, and an adjuvant comprising a refined detoxified endotoxin, and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose dimycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

27. A vaccine as defined in Claim 26 further including a pharmaceutically acceptable carrier.

28. An immunotherapeutic melanoma tumor vaccine comprising: a melanoma cell lysate of allogeneic melanoma tumor cells produced from a cell line selected from the group consisting of cell line MSM-M-1 and cell line MSM-M-2 and mixtures thereof; and an adjuvant comprising a refined detoxified endotoxin and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose imycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

29. A process for the treatment of a melanoma tumor on a patient, which comprises administering to said patient an anti-tumor effective amount of a vaccine comprised of a melanoma cell lysate of allogeneic melanoma tumor cells produced from a cell line selected from the group consisting of cell line MSM-M-1 and cell line MSM-M-2 and mixtures thereof, and an adjuvant comprising a refined detoxified endotoxin, and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose dimycolate pyridine soluble extract of a microorganism, and mixtures thereof.

30. A process for the treatment of a melanoma tumor in a host, which comprises administering to said host an anti-tumor effective amount of a vaccine comprised of a melanoma cell lysate of allogeneic melanoma tumor cells produced from a cell line selected from the group consisting of cell line MSM-M-1 and cell line MSM-M-2, and mixtures, thereof, and an adjuvant comprising a refined detoxified endotoxin, and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose dimycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

31. A vaccine useful for the treatment and prevention of melanoma tumors in a host, said vaccine comprising a melanoma cell lysate produced from an in vitro culture of allogeneic melanoma tumor cells selected from the group consisting of cell line MSM-M-1 and cell line MSM-M-2 and mixtures thereof, and an adjuvant comprising a refined detoxified endotoxin, and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose dimycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

32. A vaccine as defined in Claim 31 further including a pharmaceutically acceptable carrier.

33. An immunotherapeutic melanoma tumor vaccine composition characterized by a melanoma cell lysate of allogeneic melanoma tumor cells produced from a cell line selected from the group consisting of cell line MSM-M-1 and cell line MSM-M-2, and mixtures thereof, and an immunologically effective amount of an adjuvant comprising a refined detoxified endotoxin, and at least one biological immunostimulant selected from the group consisting of mycobacterial cell wall skeleton, trehalose dimycolate, pyridine soluble extract of a microorganism, and mixtures thereof.

34. The cell line MSM-M-1.

35. The cell line MSM-M-2.

36. A melanoma cell lysate of melanoma tumor cells produced from the cell line MSM-M-1.

37. A melanoma cell lysate of melanoma tumor cells produced from the cell line MSM-M-2.

38. A melanoma cell lysate of melanoma tumor cells produced from the cell lines MSM-M-1 and MSM-M-2.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04139

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. 4th Edition A61K 39/00; A61K 39/02; C12N 5/00

U.S. CL. 424/88; 424/92; 435/240.2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/88; 424/92; 435/240.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

ONLINE SEARCH CONDUCTED ON APS, CA AND BIOSIS.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X, P	Cancer Research, Volume 48, issued 15 October 1988, Mitchell et al., "Active specific immunotherapy for melanoma: Phase I trial of allogenic lysates and a novel adjuvant". See pages 5883-5892.	1-38
X, E / Y	US, A, 4,877,611, Cantrell, published 31, October 1989, see the entire document.	1-34 / 1-34
Y	US, A, 4,436,727, Ribi, published 13 March 1984, see the entire document.	1-33
Y	US, A, 4,505,900, Ribi, published 19 March 1985, see the entire document.	1-33

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

6 Dec. 1989

Date of Mailing of this International Search Report

06 JAN 1990

International Searching Authority

ISA/US

Signature of Authorized Officer

Abdel A. Mohamed

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Biological Response Modifiers, Volume 5, No. 3, issued 1986, Bystryn et al., "Preparation and characterization of a polyvalent human melanoma antigen vaccine" see pages 211-223.	1-33
A,P	US, A, 4,806,352, Cantrell, published 21 Feb. 1989, see the whole document.	1-33
A	Cancer, volume 44, issued August 1979, Vosika et al, "Intralesional immunotherapy of malignant melanoma with Mycobacterium smegmatis cell wall skeleton combined with trehalose dimycolate (P_3)". See pages 495-502.	1-33
A	The Journal of Immunology, volume 138, No. 5, issued 1 March 1987, Livingston et al, "Approaches to augmenting the immunogenicity of the ganglioside GM2 in mice: purified GM2 is superior to whole cell." See the entire document, 1524-29.	1-33